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☐ 1. Document ID: US 6383496 B1

L5: Entry 1 of 1 File: USPT May 7, 2002

DOCUMENT-IDENTIFIER: US 6383496 B1
TITLE: Recombinant vaccines comprising immunogenic attenuated bacteria having RPOS positive phenotype

US PATENT NO. (1):
6383496

Detailed Description Text (54):
The bacterial strains used in the present studies were constructed using the following general materials and methods. Listings of phages, plasmids and micro-organisms used in constructing the strains are given in Tables 1 and 2.

Detailed Description Text (58):
Suicide vectors containing an ampicillin-resistance gene, a sucrose-utilization cassette, and an incP mobilization site were constructed. Mutant genes which have been introduced into these plasmids can be introduced into the bacterial chromosome after transformation, or preferably by conjugation, to generate ampicillin-resistant (100 .mu.g/ml) merodiploids. Such merodiploids can be grown on media containing 5% sucrose to select for the loss of the integrated plasmid along with the ampicillin-resistance and sucrose-utilization genes. Ampicillin-sensitive strains can be phenotypically characterized for the presence of appropriate defined deletion mutant alleles.

Detailed Description Text (82):
To determine whether the ropS gene product regulates expression of chromosomally-encoded genes whose products are important for S. typhimurium colonization of Peyer's patches, the wild-type .chi.3339 and rpoS mutant .chi.4973 strains were cured of their virulence plasmids to generate plasmid-cured isogenic derivatives .chi.3340 and .chi.8125, respectively. The ability of these derivative strains to colonize Peyer's patches was examined following peroral administration of .chi.3340 and .chi.8125 in a 1:1 ratio and the data are shown in Table 7 below.

Detailed Description Text (98):
For example, genomic DNA libraries from wild-type Salmonella typhimurium UK-1 (.chi.3761) can be constructed in a suitable cloning vector such as pNEB-193 (New England Biolabs), which is a pUC19 derivative that carries single sites for the unique 8-base cutters: AscI, PacI and PmeI. Generally, genomic DNA is isolated according to standard methods (Sambrook et al., Molecular Cloning/A Laboratory Manual Second

Edition, Cold Spring Harbor Press, Cold Spring Harbor, N.Y., 1989). Sau3A1 partially digested genomic DNA is sized on an agarose gel and extracted using commercially available methods in kit form obtained from Promega, Quiagen, or Bio101. DNA fragments between 2 and 6 kb are isolated and ligated into a plasmid first digested with BamHI or BglII, then dephosphorylated using alkaline phosphatase according to the manufacturers' instructions. The resulting plasmid library is then introduced into an appropriate E. coli strain in order to amplify the genomic library and to obtain a population of recombinant plasmids containing random genomic DNA inserts ranging in size from 2 to 6 kb. Relevant clones are isolated from a genomic library by complementation of mutant E. coli or S. typhimurium strains.

Detailed Description Text (121):
The construction of the defined .DELTA.phoPQ23 .DELTA.asdA16 S. typhi strains in both the ISP1820 and Ty2 backgrounds involved the use of two suicide plasmids, pMEG-213 containing the .DELTA.phoPQ23 region and pMEG-006 containing the .DELTA.asdA16 region.

Detailed Description Text (178):
The wild-type rpoS gene can be introduced into the chromosome of .chi.3769, MGN-1018 or .chi.8280 by allelic exchange using the suicide properties of the R6K-based plasmid pMEG-149 or its derivative pMEG-375. Plasmids pMEG-149 and pMEG-375 are mobilizable suicide vectors which carry a .lambda.pir-dependent R6K replicon and thus require a host with the pir gene present in trans to allow replication. In addition, pMEG-149 encodes the selectable marker for Ap.sup.r and the counterselectable marker, levanosucrase whereas pMEG-375 also contains the cat gene specifying resistance to chloramphenicol (Cm.sup.r). Since pMEG-149 and pMEG-375 cannot replicate in strains lacking the pir gene, selection of Ap.sup.r and Ap.sup.r Cm.sup.r transconjugants, respectively, demands the integration of the plasmid into the chromosome, an event which usually takes place through homology in the inserted fragment.

Detailed Description Text (188):
The rpoS.sup.+ vaccine strains are prepared based upon S. typhi strains containing a functional rpoS gene such as ISP1820 using defined deletions as described above in examples 2 and 3 or based upon attenuated rpoS mutant strains such as Ty2 which have a recombinant rpoS gene as described in example 10 above. In the construction of vaccines expressing foreign antigens, the preferred approach is to use a balanced, lethal host-vector system which confers stable maintenance and high-level expression of cloned genes on recombinant plasmids. For this, a chromosomal mutation of the asd gene encoding aspartate .beta.-semialdehyde dehydrogenase is introduced into the RpoS.sup.+ strain to impose an obligate requirement for diaminopimelic acid (DAP) which is an essential constituent of the rigid layer of the bacterial cell wall and which is not synthesized in humans. The chromosomal .DELTA.asd mutation is then complemented by a plasmid cloning vector possessing the wild-type asd.sup.+ gene as well as a recombinant gene encoding the desired foreign antigen. Loss of the plasmid results in DAP-less death and cell lysis. Such balanced-lethal host-vector combinations are stable for several weeks in the immunized animal host and elicit immune responses against the cloned gene product as well as against Salmonella.

Detailed Description Text (245):
Bacteriology: Stools and rectal swabs will be inoculated into selenite-cystine broth. Stools must be processed within 48 hours. After overnight incubation at 37.degree. C., subcultures will be made onto XLT-4 agar. Colonies which appear consistent with Salmonella will be processed through API-20 system of identification and confirmation made by a agglutenation with S. typhi O, H, and Vi antisera. These isolates will be saved at -70.degree. C. in 5% glycerol-1% peptone for further analysis (e.g., for the presence of plasmids, for absence or presence of specific DNA sequences using PCR, or for Southern blotting with gene probes for cloned genes).

Detailed Description Paragraph Table (2):

TABLE 2 Phages and Plasmids Bacteriophage Description Source/Reference P22HTint high frequency Schmeiger, Mol. Gen. Genet. 119:75-88, 1972; Jackson generalized transducing et al., J. Mol. Biol. 154:551-563, 1982; Ray et al., mutant of the temperate Mol. Gen. Genet. 135:175-184, 1974. lambdoid phage P22 P22 H5 clear plaque forming Casjens et al., J. Mol. Biol. 194:411-422, 1987. mutant of P22HTint Plasmids pSK::rpoS S. typhimurium 14028 F. Fang, Univ. Colorado Health Sci. Center rpoS.sup.+ gene cloned into the EcoRV site of pBlueScript/SK pMEG-003 pir-dependent R6K ori MEGAN Health, Inc., St. Louis, MO Tc.sup.r asd.sup.+ pMEG-006 pir-dependent R6K ori Megan Health, Inc., St. Louis, MO Tc.sup.r .DELTA.asdA16 pMEG-068 Contains phoQ gene MEGAN Health, Inc., St. Louis, MO pMEG-149 Amp.sup.R mobilizable pir- MEGAN Health, Inc., St. Louis, MO dependent suicide vector; containing the sacBR genes from B. subtillis, RK2 mob, R6K ori pMEG-210 phoQ deletion of pMEG- MEGAN Health, Inc., St. Louis, MO 068 pMEG-213 Derivative of pMEG-149 MEGAN Health, Inc., St. Louis, MO containing phoPQ23 defined deletion of pMEG-210 pMEG-328 Derivative of pNEB-193 MEGAN Health, Inc., St. Louis, MO containing the S. typhimurium UK-1 rpoS.sup.+ gene cloned into the Bam HI and XbaI sites pMEG-375 cat gene from pACYC184 MEGAN Health, Inc., St. Louis, MO cloned into PMEG-149 pNEB-193 pUC19 derivative that New England Biolabs carries single restriction sites for unique 8bp cutters AscI, PacI and PneI within the polylinker region pYA3167 asd - complementing Nardelli-Haefliger et al., Infect. Immun. 64:5219- plasmid; expresses the 5224, 1996 Hepatitis B virus (HBV) nucleocapsid pre-S1 and pre-S2 epitopes on HBV core pYA3342 Asd.sup.+ cloning vector This lab with pBR replicon pYA3433 contains rpoS.sup.+ gene This lab cloned from pSK::rpoS into SmaI site of pMEG- 149 pYA3467 contains S. typhimurium This lab UK-1 rpoS.sup.+ gene from pMEG-328 cloned into PmeI and Sma I sites of pMEG-375

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Mol Microbiol 1994 Aug;13(4):733-43

Related Articles, Links

The nlpD gene is located in an operon with rpoS on the Escherichia coli chromosome and encodes a novel lipoprotein with a potential function in cell wall formation.

Lange R, Hengge-Aronis R.

Department of Biology, University of Konstanz, Germany.

rpoS is the structural gene for sigma s, which is a second vegetative sigma subunit of RNA polymerase in Escherichia coli and is involved in the expression of many stationary phase-induced genes. Upstream of rpoS is an open reading frame (ORF) whose function and regulation have not been studied. Strong overproduction of its gene product using the IPTG-inducible tac promoter leads to the formation of bulges at the cell septum and the cell poles, and in rapidly growing cells brings about

cell lysis, indicating that the gene product has a hydrolytic function in cell wall formation or maintenance. This is corroborated by sequence homology to lysostaphin, a cell wall lytic exoenzyme synthesized by two Staphylococcus strains. Using globomycin, a specific inhibitor of signal peptidase II, we demonstrate that the product of the ORF is a novel lipoprotein (NlpD). Two transcriptional start sites for nlpD have been localized. In contrast to rpoS, nlpD is not induced during entry into stationary phase. Growth-phase-regulated transcription of rpoS is initiated at additional sites within the nlpD ORF, but the nlpD promoters contribute substantially to the basal level of rpoS expression in exponentially growing cells, indicating that nlpD and rpoS form an operon.

PMID: 7997184 [PubMed - indexed for MEDLINE]

Mutat Res 1988 Sep;194(2):131-41

Related Articles, Links

A mutation in the DNA adenine methylase gene (dam) of Salmonella typhimurium decreases susceptibility to 9-aminoacridine-induced frameshift mutagenesis.

Ritchie L, Podger DM, Hall RM.

CSIRO Division of Molecular Biology, North Ryde, NSW, Australia.

A mutant of Salmonella typhimurium with a reduced response to mutation induction by 9-aminoacridine (9AA) has been isolated. The mutation (dam-2) is located in the DNA adenine methylase gene. The dam-2 mutant strain exhibits a level of sensitivity to 2-aminopurine (2AP) intermediate between that of the dam+ and the DNA adenine methylation-deficit dam-1 strain, and 2AP sensitivity was reversed by introduction of a mutH mutation or of the plasmid pMQ148 (which carries a functional

Escherichia coli dam+ gene). However, the dam-2 strain is not grossly defective in DNA adenine methylase activity. Whole cell DNA appears full methylated at -GATC- sites. The levels of 9AA required to induce equivalent levels of frameshift mutagenesis in the dam-2 strain were approximately 2-fold higher than for the dam+ strain. Introduction of pMQ148 dam+ reduced the level of 9AA required for induction of frameshift mutations 4-fold in the dam-2 strain and 2-fold in the dam+ strain. The dam-2 mutation had no effect on the levels of ICR191 required for induction of frameshift mutations, but introduction of pMQ148 reduced the ICR191-induced mutagenesis 2-fold. The dam+/pMQ148, dam-2/pMQ148 and dam-1/pMQ148 strains showed identical dose-response curves for both 9AA and ICR191. These results are consistent with a slightly reduced (dam-2) or increased (pMQ148) rate of methylation at the replication fork. The 2AP sensitivity of the dam-2 strain cannot be simply explained. Furthermore, addition of methionine to the assay medium reverses the 2AP sensitivity of the dam-2 strain, but has no effect on 9AA mutagenesis.

PMID: 2842672 [PubMed - indexed for MEDLINE]

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☐ 1. Document ID: US 6383496 B1

L3: Entry 1 of 1 File: USPT May 7, 2002

DOCUMENT-IDENTIFIER: US 6383496 B1
TITLE: Recombinant vaccines comprising immunogenic attenuated bacteria having RPOS positive phenotype

US PATENT NO. (1):
6383496

Brief Summary Text (26):
As noted above, the delivery of a polynucleotide encoding the desired gene product to a human is within the scope of the methods and compositions.of the present invention. Moreover, each of the embodiments above involving methods and compositions based upon microbes having an RpoS.sup.+ phenotype are further contemplated to include methods and compositions for the delivery of a gene or portion thereof to the cells of a human. The gene or portion thereof can comprise a eukaryotic expression cassette that contains the genetic information, either DNA or RNA, that is intended to be delivered to cells of the human.

Brief Summary Text (27):
Thus, in one embodiment of the present invention provides methods for delivery of a gene or portion thereof to the cells of a human. One such method comprises selecting a strain of bacteria such as S. typhi on the basis of the strain having (i) an RpoS.sup.+ phenotype, (ii) one or more inactivating mutations which render the strain attenuated, and (iii) the gene or portion thereof. The gene or portion thereof can be within a eukaryotic expression cassette. The selecting step with respect to RpoS.sup.+ phenotype can involve, in whole or in part, testing the strain to determine its RpoS phenotype. The method can also comprise delivering to cells of a human, a live attenuated strain of bacteria having (a) an RpoS.sup.+ phenotype, (b) a recombinant rpoS.sup.+ gene, (c) one or more inactivating mutations which render said microbe attenuated and (d) the gene or portion thereof. The gene or portion thereof can be within a eukaryotic expression cassette.

Brief Summary Text (28):
The present invention also provides methods for producing a strain of carrier microbes for delivery of a desired gene or portion thereof to a cell of a human. One such method can comprise (1) selecting for a strain of S. typhi or other bacteria having an RpoS.sup.+ phenotype; (2) producing one or more inactivating mutations in the RpoS.sup.+ strain to render the strain attenuated; and (3) introducing into the strain the gene or portion thereof. The gene or portion thereof can be within a eukaryotic expression cassette. The selecting step can involve, in whole or in part, testing the strain to determine its RpoS phenotype and the steps can be performed in any order. The method can also comprise generating a live attenuated strain of S. typhi or other

bacteria having (a) an RpoS.sup.+ phenotype, (b) a recombinant rpoS.sup.+ gene, (c) one or more inactivating mutations which render said microbe attenuated and (d) the desired gene or portion thereof. The gene or portion thereof can be within a eukaryotic expression cassette.

Detailed Description Text (25):
The attenuated microbes of this invention can additionally be used as vectors for the synthesis of various host proteins. Because the attenuated microbes of this invention are able to traverse a variety of immunocompetent structures including gut-associated lymphoid tissue (GALT), mesenteric lymph nodes and spleen after introduction into the host, such microbes can be used to target a variety of immunoregulatory products. Accordingly, one or more genes encoding immunoregulatory proteins or peptides can be recombinantly introduced into the attenuated microbes such that when the microbes taking up residence in the appropriate immunocompetent tissue are capable of expressing the recombinant product to suppress, augment or modify the immune response in the host. Examples of immunoregulatory molecules include but are not limited to: colony stimulating factors (macrophage, granulocyte, or mixed), macrophage chemotoxin, macrophage inhibition factor, leukocyte inhibitory factors, lymphotoxins, blastogenic factor, interferon, interleukins, tumor necrotizing factor, cytokines, and lymphokines.

Detailed Description Text (27):
The recombinant gene of the microbes of the present invention can be incorporated into a "balanced-lethal" system which selects for microorganisms containing and capable of expressing the recombinant gene by linking the survival of the microorganism to the continued presence of the recombinant gene. "Balanced-lethal" mutants of this type are characterized by a lack of a functioning native chromosomal gene encoding an enzyme which is essential for cell survival, preferably an enzyme which catalyzes a step in the biosynthesis of diaminopimelic acid (DAP) and even more preferably a gene encoding beta aspartate semialdehyde dehydrogenase (Asd). DAP pathway enzymes and Asd are required for cell wall synthesis. The mutants also contain a first recombinant gene which can serve to complement the non-functioning chromosomal gene and this is structurally linked to a second recombinant gene encoding the desired product. Loss of the complementing recombinant gene causes the cells to die by lysis when the cells are in an environment where DAP is lacking. This strategy is especially useful since DAP is not synthesized by eukaryotes and, therefore, is not present in immunized host tissues. Methods of preparing these types of "balanced lethal" microbes are disclosed in U.S. Pat. No. 5,672,345.

Detailed Description Text (40):
Nucleic acid vaccines are well known in the art (see e.g., Ulmer et al., Amer. Soc. Microbiol. News 62:476-479, 1996; Ulmer et al., Curr. Opinion. Immunol. 8:531-536, 1996; and Robinson, H. L., Vaccine 15:785-787, 1997) and delivery of DNA vaccines by attenuated bacteria with subsequent stimulation of an immune response against the protein-encoded by the DNA vaccine has been described (Sizemore et al., Vaccine 15:804-806, 1997). Thus, it is expected that the attenuated microbes of the present invention can also be used as delivery vehicles for DNA vaccines. Typically, bacteria containing such DNA vaccines do not themselves express the gene product encoded by the DNA vaccine, but release the DNA vaccine into one or more human tissues, where the gene product is then expressed by host cell transcription and translation machinery. However, it is also contemplated that a DNA vaccine for immunization against RNA viruses can be constructed in which copies of the RNA viral genome, or of a protein-encoding portion thereof, will be made in the cytoplasm of the attenuated bacteria. Such RNA molecules would be released into the human tissues, e.g., by lysis of the attenuated bacteria, where they would serve as mRNA for synthesis of immunogenic viral protein(s). It is also contemplated that the DNA vaccine vector within the attenuated bacterial host could synthesize the mRNA for a desired gene product within the bacteria which could then be delivered to the eukaryotic cell where the mRNA would be directly translated into the desired gene product. In this case, the mRNA would possess information or signals that caused translation to be dependent on the eukaryotic host cell and which would preclude, for the most part, translation within the attenuated bacterial cell.

Detailed Description Text (195):
It is well known that live attenuated bacterial vaccines induce long-lasting immunity by inducing T helper lymphocyte memory functions. S. typhimurium infection of mice leads predominantly to a Th-1 type of response although a Th-2 response with production of SIgA in mucosal secretions and serum antibodies against Salmonella and against foreign expressed antigens is also induced. IL-10 can be detected at levels indicating the occurrence of the Th-2 response (Van Cott et al., J. Immunol. 156:1504-1514, 1996). It also known that the recombinant attenuated S. typhimurium vaccine can also induce a CTL response involving CD-8.sup.+ cells against a foreign antigen (Sadoff et al., Science 240:336, 1988). In many cases, however, it would be desirable if a recombinant attenuated Salmonella vaccine elicits predominantly a Th-2 type of response to enhance mucosal immunity by the production of SIgA and a cellular memory response for that SIgA production. The lymphokines IL-4 and IL-5 when produced, potentiate such a Th-2 response. On the other hand, it is desirable in other instances to maximize the ability of the recombinant attenuated Salmonella to induce a Th-1 type of response which might be particularly important in providing protective immunity against a facultative or obligate intracellular parasite whose antigens are expressed by the recombinant attenuated Salmonella vaccine. Shifting the immune response to a predominantly Th-1 or to a Th-2 type of response can be achieved in part by expressing lymphokines via recombinant attenuated Salmonella strains. Thus, we have constructed Salmonella strains expressing IL-2 which enhances the Th-1 type of response and also potentiates a CTL response which is important in designing attenuated Salmonella vaccines to be protective in combating certain types of cancer (Saltzman et al., Cancer BioTher. Radiol. Pharm. 11:145-153, 1996; Saltzman et al., J. Pediatric Surg. 32:301-306, 1997). Generating the Salmonella to induce a predominant Th-2 response can be achieved by causing the strains to express IL-4 and IL-5 as has been done for the latter lymphokine by Whittle et al. (1997, J. Med. Microbiol. 46:1029-1038). IL-4 has been expressed by a recombinant aroA attenuated Salmonella vaccine strain but was not effective since it was not secreted (Denich et al., Infect. Immun. 61:4818-4827, 1993). Methods such as described by Hahn et al. (FEMS Immunol. Med. Microbiol. 20:111-119, 1998) are now available to succeed in such secreted expression of lymphokines by attenuated Salmonella. It is also possible to coexpress peptides such as factor P which is reported to stimulate the secretion of SIgA. Genes for cDNAs have been obtained which specify many different lymphokines, cytokines and other peptide or protein molecules which act to modulate the immune response. It is anticipated that these peptides or proteins could be coexpressed by recombinant attenuated Salmonella vaccine strains expressing some antigen from a particular pathogen or from a tumor cell line or some other molecule that was targeted for an immune response that would induce an immune response to protect against an infectious disease or to therapeutically correct against a systemic disease of the immunized human. Thus IL-6 has been expressed and in some cases secreted by recombinant attenuated Salmonella (Dunstan et al., Infect Immun 64:2730-2736, 1996; Hahn et al., FEMS Immunol Med Microbiol 20:111-119, 1998). The genes for murine macrophage inhibitory factor (MIF), IL-2, IFN-.gamma. or TNF-.alpha. were individually cloned and expressed by recombinant attenuated Salmonella to alter immune responses against Leishmania major infection (Xu et al., J. Immunol. 160:1285-1289, 1998). TGF-.beta. has also been expressed in recombinant attenuated Salmonella vaccine strains to decrease the inflammatory response by inhibiting endogenous synthesis of IL-2 and INF-.gamma. but enhancing synthesis of IL-10 (Ianaro et al., Immunology 84:8-15, 1995). Based on data presented in preceding examples, it is evident that recombinant attenuated Salmonella vaccines of the RpoS.sup.+ phenotype will be superior to vaccine strains of an RpoS.sup.- phenotype in expressing cytokines and other immunoactive molecules to suppress, enhance and/or modulate the immune response in a desired way.

Detailed Description Text (197):
This example illustrates methods which can be used in constructing recombinant attenuated vaccine strains to combat cancer by supressing tumor growth, by enhancing the immunized individuals immune system to eliminate tumor cells and/or by inducing an immune response against a tumor-specific antigen.

Detailed Description Text (198):
As stated in Example 12 above, we have constructed Salmonella strains expressing IL-2 which enhances the Th-1 type of response and also potentiates a CTL response which has been effective in decreasing metastases by murine adenocarcinoma MC-38 (Saltzman et al., 1996: Saltzman et al, 1997). Based on results presented, it is evident that a recombinant attenuated Salmonella vaccine designed to supress tumor growth and spread would be more efficacious if displaying an RpoS.sup.+ phenotype rather than a RpoS.sup.- phenotype.

Detailed Description Text (199):
It is known that Salmonella will seek out and partially destroy solid tumors following infection of a tumor-bearing individual. (Pawelek et al., Cancer Res 57:4537-4544, 1997). Such wild-type Salmonella ultimately kill the host as well as destroy the tumor. It is, therefore, necessary to attenuate the Salmonella and also to modify it to eliminate the inflamatory response resulting from the induction of TNF.alpha. by the lipid A moiety of LPS. This has been accomplished by using a purine-requirement mutant that is attenuated with an inactivated msbB gene which renders the lipid A non-inflamatory (Low et al, Nature Biotechnol. 17:37-41, 1999). Such strains can be further modified by introduction of a .DELTA.asd mutation and endowed with an Asd.sup.+ plasmid vector specifying an enzyme that converts a non-toxic prodrug into an anti-tumor drug within the tumor to further enhance the rate of tumor destruction (see, for example, WO9913053). Based on results presented, it is evident that a recombinant attenuated Salmonella vaccine designed to supress tumor growth and spread would be more efficacious if displaying an RpoS.sup.+ phenotype rather than a RpoS.sup.- phenotype.

Detailed Description Text (200):
The attenuated S. typhi strains with the balanced-lethal host-vector system as described in Example 11 can also be used to express tumor-specific antigens to create a therapeutic anti-cancer vaccine. Such vaccines would thus be endowed with the ability to express a specific tumor-specific antigen often fused to a T-cell epitope to enhance induction of a CTL response. Such a vaccine could also be enhanced by secreting IL-2 and rendered less inflamatory by introducing a msbB mutation or one of similar effect in depressing the inflamatory effect of lipid A. Based on results presented, it is evident that a recombinant attenuated Salmonella vaccine designed to supress tumor growth and spread would be more efficacious if displaying an RpoS.sup.+ phenotype rather than a RpoS.sup.- phenotype.

Detailed Description Text (260):
Sizemore et al. (Science, 270:299-302, 1995; Vaccine 15:804-807, 1997) described the use of Shigella flexneri 2a strain 15D with a .DELTA.asd mutation that harbored a DNA vaccine vector engineered to express E. coli .beta.-galactosidase. The Shigella strain was attenuated due to the .DELTA.asd mutation which causes death due to absence of diaminopimelic acid upon invasion into eukaryotic cells. The strain was able to deliver the DNA vaccine vector intracellularly after attachment to, invasion into and lysis within the cytoplasm of eukaryotic cells in culture or within immunized mice. More recently, others have used S. typhimurium strains possessing a DNA vaccine vector and caused to lyse by spontaneous means (Powell et al., WO96/34631, 1996; Pasenal et al., Behring. Inst. Mitt. 98:143-152, 1997; Darji et al., Cell 91:765-775, 1997). In cases in which lysis was spontaneous, it was necessary that the bacterial strain possess one or more deletion mutations rendering the strain attenuated. Shigella, Salmonella and invasive E. coli are known to have a much enhanced ability to attach to and invade M cells overlying the GALT rather than to attach to and invade intestinal epithelial cells (enterocytes). Delivery of foreign antigens or the production of foreign antigens within the NALT, BALT, CALT and GALT which all have an M cell layer leads to induction of mucosal immune responses as well as systemic immunity. Because mucosal immune responses are protective against the vast majority of infectious disease agents that colonize on or invade through a mucosal surface, it would be expected that DNA vaccine vectors could thus be delivered by RpoS.sup.+ Salmonella, Shigella, Escherichia or hybrids between any two of these genera. These microbes would have a superior ability to attach to and invade the M cells overlying the lymphoid tissues of the NALT, CALT, BALT and GALT. Because both oral and intranasal

immunization with RpoS.sup.+ microbes increase the immune response, it would be expected that attenuated bacterial DNA vaccine vector strains displaying an RpoS.sup.+ phenotype will give an increased immune response when administered intranasally or perorally and presumably by other routes that stimulate mucosal immune responses.

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